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明　細　書

1. 発明の名称

抗ストレプトリジンOの新規測定法

2. 特許請求の範囲

(1) 標識物質を内包したリポソームと抗原であるストレプトリジンOを用い、リポソーム膜がストレプトリジンOにより破壊されて放出される標識物質の量を測定することにより抗ストレプトリジンO（ASLO）価を測定することを特徴とする、ASLO価の測定法。

(2) 標識物質が酵素、補酵素、発光性化合物、螢光性化合物、色素、糖類、イオン性化合物、ラジカル性化合物である、特許請求の範囲第1項記載の測定法。

3. 発明の詳細な説明

〔産業上の利用分野〕

本発明は、リポソームを用いる抗ストレプトリジンO（以下、ASLOと略す。）価の新規測定法に関する。

〔従来の技術及びその問題点〕

ASLO価の測定は、溶連菌感染の既往の証明の為に広く用いられている検査である。ストレプトリジンO（以下、SLOと略す。）は連鎖球菌の産生する溶血性毒素であって、抗原性を有している為に、A群溶連菌の感染が起こると種々の溶連菌体外毒素に対する抗体価が上昇するので、それを見出すことにより溶連菌感染の既往を証明できることになる。

ASLO価測定法として従来から行われている方法としては、ウサギ、ヒツジ又はヒトO型赤血球を用いるランツ・ランダール法及びマイクロタイター法がある。これらはいずれも抗原であるSLOが検体血清中の抗体であるASLOと反応してSLOが有している溶血活性や細胞破壊活性等の毒素活性を不活性化する所謂中和反応を利用してASLO価の測定を行っている。しかしながら、これらの方法では、必然的に動物やヒトの新鮮な赤血球（極めて不安定である）を必要とする為、測定値間のバラツキを生じ易く、また操作法も煩雑である。その上、反応時間も比較的長い為、自動化は極めて困

難である。

一方、一種の沈降反応を利用した免疫比濁法による測定法も開示されている（特開昭61-25062号公報等。）。しかしながら、SLO-ASLOの抗原抗体反応による濁りは、他の抗原抗体反応のそれと比べて濁りの度合が非常に小さい。その為、他の蛋白をも非特異的に沈降させてしまうような所謂凝集促進剤等の添加物を加える必要があり、系の複雑化を伴い、自動化するには問題が多く、また正確性にも欠ける。

〔発明の目的〕

本発明の目的は、赤血球のような不安定な物質を使用せず、簡便で迅速且つ正確に測定ができ、自動化が可能なASLO価の新規測定法を提供することにある。

〔発明の構成〕

上記目的を達成する為、本発明は下記の構成から成る。

「標識物質を内包したリポソームと抗原であるストレプトリジンOを用い、リポソーム膜がストレプトリジンOにより破壊されて放出される標識物質の量を測定することにより抗ストレプトリジンO（ASLO）価を測定することを特徴とするASLO価の測定法。」

即ち、本発明者らは、自動化が可能なASLO価の簡便、迅速且つ正確な測定法を求めて研究の途上、SLOがその溶血活性、細胞破壊活性に基づきリポソームを破壊する性質に着目、これをASLO価の測定に利用できないかと考え、鋭意研究を重ねた結果、本発明を完成するに到った。

本発明で用いられるリポソームとしては、従来からよく知られている、リン脂質或は糖脂質と、コレステロール及びその他の脂質との混合系のもの、或はこれらに更にリポ多糖（以下、LPSと略す。）又はLPS様化合物を加えたもの等が挙げられる。リン脂質としては通常用いられるジパルミトイルフォスファチジルコリン（DPPC），ジミリストイルフォスファチジルコリン（DMPC），ジステアロイルフォスファチジルコリン（DSPC）等のアルキルエステル系のものや、例えば、ジ－O－ヘキサデシルフォスファチジルコリン，ジ－O－オクタデシルフォスファチジルコリン，ジドデシルフォスファチジルコリン等のようなアルキルエーテル系のもの等いずれのものでもよいが、アルキルエーテル系のものは一般に常温でも安定なリポソームを形成し得るので、より好ましい。

リポソームに内包される標識物質としては、例えばアルカリフォスファターゼ，β－ガラクトシダーゼ，グルコース－6－リン酸脱水素酵素等の酵素類、例えば、NAD，NADP，FAD等の補酵素類、例えば、ルミノール，ルシフェリン等の発光性化合物、例えば、カルボキシフルオレセイン等の蛍光性化合物、例えば、2,7－ビス（2－アルソノフェニルアゾ）－1,8－ジヒドロキシナフタレン－3,6－ジスルホン酸等の色素類、例えばグルコース等の糖類、例えば、重クロム酸カリ，重クロム酸ナトリウム，NaCl等のイオン性化合物、例えば、ニトロキシド化合物等のラジカル性化合物等が挙げられるがこれらに限定されるものではなく、リポソームに封入可能で、ASLO測定に於てマーカーとなり得るものであればいずれにても良い。

本発明で用いられるリポソームの調製法としては、従来からよく知られているボルテクスイング法、超音波法、界面活性剤法、逆相蒸発法（REV法），エタノール注入法、エーテル注入法、プレ－ベジクル（Pre-Vesicle）法、フレンチプレスエクストルージョン（French Press Extrusion）法、Ca^{2+}融合法、アニーリング（Annealing）法、凍結融解融合法、凍結乾燥法、W/O/Wエマルジョン法等の方法や、最近、S.M.Gruner ら〔Biochemistry, 24, 2833(1985)〕により報告されたStable Plurilamellar Vesicle 法（SPLV法）などの方法、更には、内包量の大きな"巨大リポソーム(Giant Liposome)"と言われているリポソームを調製する方法など、自体公知のリポソームの調製法は全て挙げられる。また、LPS又はLPS様化合物を含むリポソームの調製法は、本発明者らが先に特許出願した特願昭62－123542号明細書に記載の方法に準じてこれを行えばよい。尚、LPS様化合物とは、LPSそのものに何らかの化学修飾を施した化合物で、例

えばその糖鎖部分を酸化したもの、アセチル化，サクシニル化，フタロイル化等の化学的処理を行ったものや、LPSと高い親和性をもつポリミキシンや塩基性感光色素であるカルボシアニン型色素との複合体などを言う。

本発明の測定法に於て用いられるリポソーム，標識物質，SLO等の使用量を最終反応液（反応停止液を使用する測定法に於ては、反応停止液を添加する前の反応液）中の濃度として示すと下記の如くなる。リポソームの量はリン脂質量として通常5～500 n mol/ml、好ましくは30～100 n mol/mlである。リポソームに内包される標識物質の量は標識物質の種類により異なり、一律ではないが、例えばアルカリフォスファターゼ（以下、APと略す。）の場合は、通常0.02～0.6 unit/ml、好ましくは0.1～0.2 unit/mlである。SLOの量は、リポソームの種類により変動する為、特に限定されるものではないが、通常、0.5～100 μg/mlの範囲が好ましく用いられる。また、本発明の測定法に於て、標識物質として酵素を用いる場合に使用される基質量は、使用される酵素や基質の種類により異なり一律ではないが、例えばAPを用いる場合には、基質であるp－ニトロフェニルリン酸の基質溶液中の濃度として通常0.5～10 mM、好ましくは2～5 mMである。

本発明で用いられる緩衝液は、リン酸緩衝液、ホウ酸緩衝液、グッド緩衝液（HEPES，PIPES，MES等）、Tris緩衝液等、通常用いられる緩衝液が全て使用可能である。また、添加剤として牛血清アルブミン（BSA），ゼラチン等の蛋白質や、塩、キレート剤，還元剤などを適宜添加する等は任意である。但し、SLOは水溶液中では比較的不安定であるので、SLOを安定化する為に、SLO溶液に用いられる緩衝液中にはBSA，ゼラチン等の蛋白質を添加しておくことが好ましい。その添加量は特に限定されないが、通常、緩衝液中に0.05～2.0 w/v％程度添加される。

本発明の測定法について、例えば、APを標識物質として内包したリポソームを用い、基質としてp－ニトロフェニルリン酸を用いた場合を例に挙げて述べると以下の如くなる。

即ち、先ず、検体とSLOを混合して1～10分間程度プレインキュベートし、これにリポソームを加えた後、或は検体とSLOとリポソームとを混合した後、5～30分間インキュベートし、次いでこれに基質であるp－ニトロフェニルリン酸を加えて、更に5～30分間インキュベートした後、反応停止剤（例えば、NaOH，KOH等）を加えて反応を停止させ、410 nmの吸光度を測定する。或は、検体に全試薬（即ち、SLOとリポソームと基質）を同時に作用させ、5～30分間インキュベートした後、反応停止剤を加えて反応を停止させ、410 nmの吸光度を測定する。

上記いずれの方法で行うも可であるが、操作が簡便で短時間に測定が出来るという点では後者の方法が優れている。

標識物質として酵素を用いる場合には、最終反応液を所定時間予備加温した後に、或は検体，リポソーム溶液及びSLO溶液の混液を所定時間予備加温し、更にそれに基質を加えた後に、単位時間当りの吸光度の変化量を測定することによっても、ASLOの測定を行うことができる。

本発明の測定法は用手法に限らず、自動分析装置を用いた測定法にも充分利用可能であり、容易に且つ迅速に測定を行うことができる。尚、自動分析装置を用いて測定を行う場合の試薬の組合せ等については特に制約はなく、機種に合せて、或は他の要因を考慮に入れて、最もよいと思われる試薬の組合せを適宜選定して用いればよい。

以下に参考例，実施例を挙げて本発明を更に詳細に説明するが、本発明はこれら参考例，実施例により何ら限定されるものではない。

〔実施例〕

参考例1.　リン脂質としてジ－O－ヘキサデシルフォスファチジルコリンを用いたリポソームの調製

ジ－O－ヘキサデシルフォスファチジルコリンのクロロホルム溶液（20 mM）325 μl、コレステロールのクロロホルム溶液（20 mM）325 μl及びジパルミトイルフォスファチジルグリセロールの

クロロホルム／メタノール（95：5）溶液（6mM）85 μℓを試験管に入れて混合し、ロータリーエバポレーターで溶媒を留去した。デシケーター中で2時間乾燥させた後、クロロホルムとジエチルエーテルを夫々0.5 mℓずつ加え、アルカリホスファターゼ（AP）溶液［シグマ社製5000 unit／2.5 mℓの0.01M HEPES（N－2－ヒドロキシエチルピペラジン－N'－エタンスルホン酸）緩衝液溶液、pH7.4］80 μℓを添加してボルテックスミキサーで激しく撹拌した。43～48℃の水浴中、ロータリーエバポレーターで濃縮して有機溶媒を留去し、0.01M HEPES 緩衝液1 mℓを加えてボルテックスミキサーで均一に分散するまで撹拌した。これを遠沈管に移し、4℃、34000 rpmで40分間×5回超遠心処理を繰り返して上清を除き、ペレットに0.01M HEPES 緩衝液2 mℓを加えて懸濁させ、4℃で保存した。

参考例2.　LPS－リポソームの調製

参考例1に於ける脂質溶液に、新たにリポ多糖（LPS）0.5 mgをクロロホルム／メタノール（1：1）液5 mℓを加えて懸濁させ、4℃で保存した。

参考例4.　LPS－卵黄レシチン－リポソームの調製

参考例3に於けるDPPCを卵黄レシチン（1.4wt%）に代え、透析時の温度を50℃から室温に代えた以外は参考例3と全く同様にして、APを内包したリポソームを調製した。

参考例5.　LPS－DPPC－卵黄レシチン－リポソームの調製

参考例3に於けるDPPCを卵黄レシチン（1.4wt%）1.5 mℓ及びDPPC（20 mM）0.5 mℓに代え、AP水溶液量を250 μℓから1.5 mℓに代えた以外は参考例3と全く同様にして、APを内包したリポソームを調製した。

実施例1.

参考例1で得たリポソーム懸濁液10 μℓに各種濃度のASLO溶液20 μℓ、SLO溶液（濃度1 mg／mℓ）20 μℓ及び2 mM p－ニトロフェニルリン酸二ナトリウム溶液400 μℓを加え、37℃で30分間インキュベーションを行った後、0.1N NaOH 溶液1.5 mℓ

1 mℓに懸濁させた液を加えた以外は参考例1.と全く同様にして、APを内包したLPS－リポソームを調製した。

参考例3.　LPS－DPPC－リポソームの調製

ジパルミトイルフォスファチジルコリン（DPPC）のクロロホルム溶液（20 mM）2 mℓとコレステロールのクロロホルム溶液（20 mM）2 mℓを丸底フラスコに入れて混合し、ロータリーエバポレーターで溶媒を留去した。更に真空ポンプで、約2時間デシケーター中で真空乾燥を行い、脂質の薄膜をフラスコ壁面に作製した。この薄膜をLPS 0.6 mg／mℓを含むn－オクチルグルコシド（200 mM）0.6 mℓ及び0.01M HEPES 緩衝液（pH7.4）を加えて水和させ、ボルテックスミキサーで均一に分散するまで撹拌した。その後AP水溶液（2000 unit／mℓ）250 μℓを添加して、しばらく撹拌混合した後、50℃で0.01M HEPES 緩衝液に対して透析した。約2時間後、リポソームの懸濁液を遠沈管に移し、4℃、35000 rpmで40分間×5回超遠心処理を繰り返して上清を除き、ペレットに0.01M HEPES 緩衝

を加えて反応を停止し、410 nm の吸光度を測定した。結果を第1図に—○—で示す。

実施例2.

参考例2で得たLPS－リポソーム懸濁液10 μℓを用いた以外は実施例2と全く同様にして測定を行い、第1図に—●—で示す如き結果を得た。

実施例3.

各種濃度のASLO溶液20 μℓに参考例3で得たリポソーム懸濁液20 μℓを加えて50 mM Tris［トリス（ヒドロキシメチル）アミノメタン］－HCℓ緩衝液（pH7.8）で200 μℓに希釈したものに、SLOの所定濃度溶液を20 μℓ加え、更に2 mMのp－ニトロフェニルリン酸溶液400 μℓを加えた。37℃で20分間インキュベーションを行った後、0.1N NaOH 溶液2 mℓを加えて反応を停止し、410 nm の吸光度を測定した。ASLO価（Todd単位）と相対吸光度（ASLO無含有試料により得られる吸光度を100とした時の値）(%)との関係を第2図に示す。尚、第2図に於て、—●—は1 mg／mℓのSLO溶液を使用した場合の、—○—は0.5 mg／mℓのSLO溶液

を使用した場合の、—△—は0.25㎎/㎖のSLO溶液を使用した場合の結果を夫々示す。

実施例4.

参考例4で得たリポソーム懸濁液10μℓを50mM Tris－HCℓ緩衝液（pH 7.8，0.1％BSA-ゼラチン含有）で20倍希釈したもの200μℓに、同じ緩衝液で希釈したSLOのp－ニトロフェニルリン酸二ナトリウム溶液400μℓ（SLO量：2μg）を加え、37℃で30分間インキュベーションを行った後、0.1N NaOH溶液2㎖を加えて反応を停止し、410nmの吸光度を測定した。結果を第3図(a)に示す。

実施例5.

リポソーム溶液及びSLOの基質溶液の、希釈緩衝液中のBSA量を変えた以外は実施例4と全く同様にして測定を行った。結果を第3図(b)，(c)及び(d)に示す。尚、(b)は0.25％BSA含有の場合を、(c)は0.5％BSA含有の場合を、(d)は1.0％BSA含有の場合の結果を夫々示す。

この結果から明らかな如く、希釈緩衝液中のBSA量によって本発明の測定法は特に影響を受けないことが判る。

実施例6.

日立7050型自動分析装置を使用してASLO価の測定を行った。

先ず検体5μℓと第1試薬R1〔参考例5で得たリポソーム懸濁液2μℓを50mM Tris－HCℓ緩衝液（0.17％BSA・ゼラチン含有）で350μℓに希釈したもの〕350μℓとを混合してブランク補正を行い、5分後に第2試薬R2（SLO量：0.5μg，基質濃度：2mM）250μℓを加え、更に5分後、415nm（700nm）での1分間当りの吸光度変化量を測定した。本法でのASLO価の検量線を第4図に示す。第4図に於て、横軸はASLO価（Todd単位）、縦軸は検体の代わりに生理食塩水を用いた場合の反応液を対照として測定した、415nmに於ける1分間当りの吸光度変化量を示す。また、本法と従来法（ランツ・ランダール法）との相関を第5図に示す。

〔発明の効果〕

以上述べた如く、本発明は、リポソームを用いるASLO価の新規測定法を提供するものであり、本発明の方法によれば、赤血球のような不安定な物質を使用する必要がなく、簡便で、迅速且つ正確な測定を行うことができ、自動化が可能な点に顕著な効果を奏する発明である。

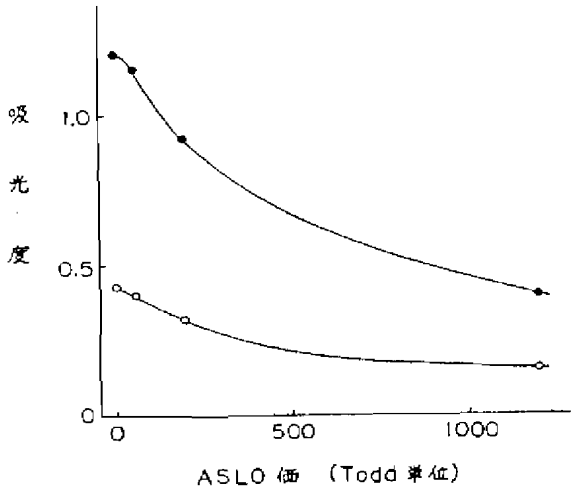
4. 図面の簡単な説明

第1図は実施例1及び実施例2で得られた検量線を示し、横軸はASLO価（Todd単位）を表わし、縦軸は410nmに於ける吸光度を表わす。但し、—○—は実施例1の、—●—は実施例2の結果を夫々示す。第2図は実施例3に於ける結果を示し、横軸はASLO価（Todd単位）、縦軸はASLO価0での吸光度（410nm）を100としたときの相対吸光度（ASLO無含有試料により得られる吸光度を100としたときの値）(%)を示す。図の—●—は1㎎/㎖のSLO溶液を使用した場合の、—○—は0.5㎎/㎖のSLO溶液を使用した場合の、—△—は0.25㎎/㎖のSLO溶液を使用した場合の結果を夫々示す。

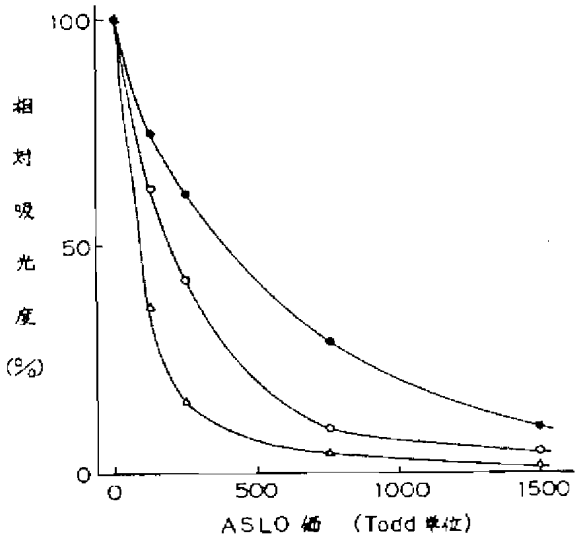
第3図(a)は実施例4，第3図(b)，(c)及び(d)は実施例5で得られた結果を夫々示し、(b)は0.25％BSA含有の場合の、(c)は0.5％BSA含有の場合の、(d)は1.0％BSA含有の場合の結果を夫々示す。図中、横軸はASLO価（Todd単位）、縦軸は410nmに於ける吸光度を夫々示す。第4図は実施例6で得られた検量線を示し、横軸はASLO価（Todd単位）、縦軸は検体の代わりに生理食塩水を用いた場合の反応液を対照として測定した、415nmに於ける1分間当りの吸光度変化量を示す。第5図は本法（実施例6の方法）と従来法（ランツ・ランダール法）との相関図を示す。

特許出願人　和光純薬工業株式会社

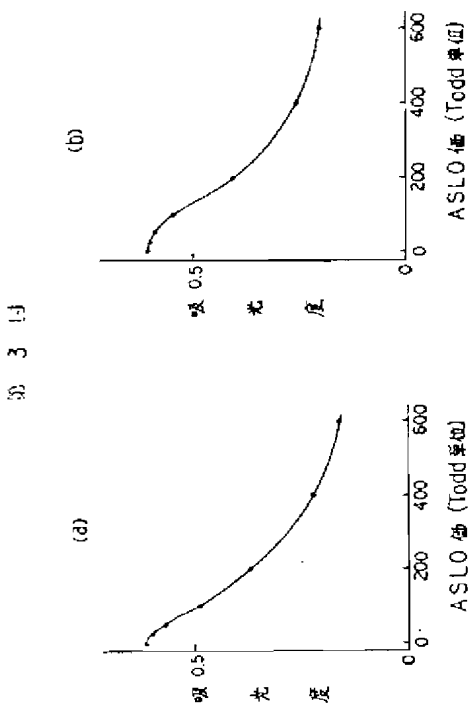
第 1 図


吸光度
1.0
0.5
0
0
500
1000
ASLO 価 (Todd 単位)

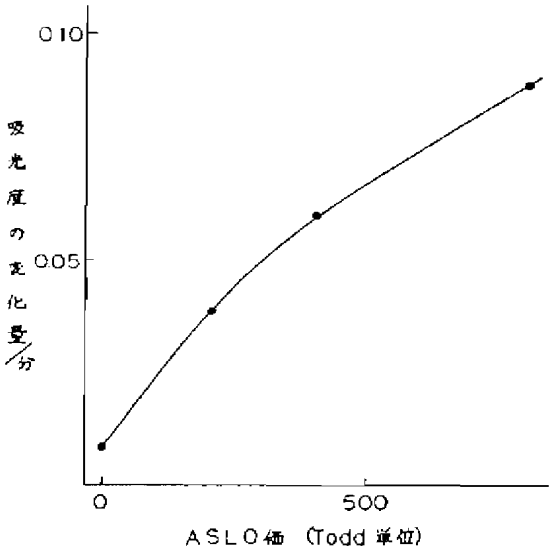
第 2 図


相対吸光度 (%)
100
50
0
0
500
1000
1500
ASLO 価 (Todd 単位)

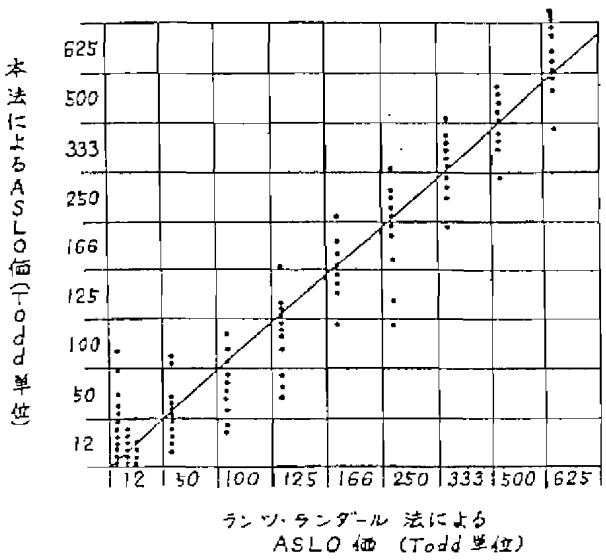
第 3 図


(a)
(b)
(c)
(d)
吸光度
0.5
0
200
400
600
ASLO 価 (Todd 単位)

第 4 図


0.10
0.05
吸光度の変化量/分
0
500
ASLO価 (Todd 単位)

第 5 図


625
500
333
250
166
125
100
50
12
本法によるASLO価(Todd単位)
12
50
100
125
166
250
333
500
625
ランツ・ランダール 法による
ASLO 価 (Todd 単位)

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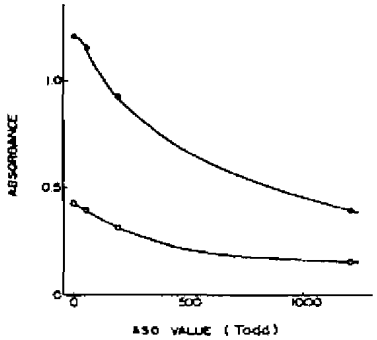
(54) Novel method for determination of antistreptolysin O and a kit used therefor.

(57) Liposomes encapsulating a labeling substance, and streptolysin O (SLO) are added to a serum sample, and after the antigen-antibody reaction between antistreptolysin O (ASO) in the sample and SLO added, the amount of the labeling substance released by lysis of the liposome membrane by the residual SLO is measured, whereby ASO value in the sample is determined. According to this determination method, ASO value can be determined rapidly and accurately and the determination can be automated.

FIG. 1



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NOVEL METHOD FOR DETERMINATION OF ANTISTREPTOLYSIN O AND A KIT USED THEREFOR

BACKGROUD OF THE INVENTION

1. FIELD OF THE INVENTION

This invention relates to a novel method for determination of antistreptolysin O (hereinafter abbreviated as ASO) value using liposomes, and a kit used therefor.

More particularly, this invention relates to a novel method for determining ASO value in a sample using liposomes encapsulating a labeling substance, and streptolysin O (hereinafter abbreviated as SLO) as antigen; and a kit used therefor. According to the novel determination method and kit of this invention, ASO value in a sample can be determined easily, rapidly and accurately and the determination can be automated.

2. RELATED ART STATEMENT

Determination of ASLO value in serum is widely used procedure for the diagnosis of previous streptococcal infection. In detail, SLO is a hemolytic toxin produced by group A streptococci and has antigenicity. Therefore, when group A-streptococcal infections occurs, antibody against SLO (that is ASO) value in serum is increased, and hence previous group A-streptococcal infections can be confirmed by detecting this increase.

Conventional methods for determining ASO value include the Rantz-Randall method and the micro-titer method which use human (O group), rabbit or sheep erythrocites. In both of these methods, erythrocytes and SLO as antigen are used. ASO value is determined by measuring the amount of erythrocytes lysed, by utilizing a so-called neutralization in which SLO reacts with SO, i.e., an antibody in a sample serum, resulting in inactivation of the toxic activities such as hemolytic activity and cytolytic activity of SLO. However, in these methods, fresh erythrocytes of an animal or a human being which is very unstable are unavaidably needed, and therefore measured values tends to scatter and a complicated procedure is required. Furthermore, the reaction time is relatively long. Accordingly, these methods are very difficult to be automated.

On the other hand a determination method by immuno-nephelometry utilizing a kind of precipitation reaction has also been reported (Japanese Patent Application Kokai (laid-Open) No. 25062/86). However, the degree of turbidity produced by the antigen-antibody reaction between SLO and ASO is much lower than that in other antigen-antibody reactions. Therefore, additives such as so-called agglutination promoters, etc. should be added. But since such additives precipitate also other proteins non-specifically, a determination system becomes to be complicated, and hence automation of said determination method involves many problems and no sufficiently accurate determination of ASO value can be achieved.

SUMMARY OF THE INVENTION

Accordingly, an object of this invention is to provide a novel determination method which uses neither unstable substance such as erythrocyte nor additive such as agglutination promoter, and permits simple, rapid and accurate determination of ASO value in a sample.

Another object of this invention is to provide a novel method for determining ASO value which can be automated.

Further another object of this invention is to provide a kit used for the novel determination method described above.

Other and further objects and advantages of this invention will be apparent from the following description.

The objects and advantages of this invention described above can be achieved by the following novel determination method and kit.

This invention is a method for determining ASO value which comprises adding liposomes encapsulating a labeling substance and SLO as both antigen and lytic agent to a sample, measuring the amount of the labeling substance released by lysis of the liposome membrane by SLO, and thereby determining ASO value in the sample; and a kit for determining ASO value which comprises a suspension of liposomes encapsulating a labeling substance, and a solution of SLO.

The determination method of this invention utilizes the ability of SLO to lyse liposomes by its hemolytic activity and cytolytic activity. More particularly, the determination method of this invention comprises adding liposomes encapsulating a labeling substance and SLO to a serum sample; measuring the amount of the labeling substance released by lysis of the liposome membrane by SLO remaining after the antigen-antibody reaction between ASO in the sample and SLO added; and determining ASO value in the sample from a standard calibration curve previously prepared by conducting determination for standard samples in the

same manner as described above.

The determination method of this invention is a simple, rapid and exact method for determining ASO value which can be automated.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the calibration curves obtained in Examples 1 and 2, the axis of abscissa refers to ASO value (Todd) and the axis of ordinate to absorbance at 410 nm. In Fig. 1 -○-shows the result obtained in Example 1 and -●-that obtained in Example 2. Fig. 2 shows the results obtained in Example 3, the axis of abscissa refers to ASO value and the axis of ordinate to relative absorbance determined by taking the absorbance (at 410 nm) at an ASO value of zero as 100 (i.e., an absorbance value determined by taking the absorbance measured for a sample containing no ASLO) (%). In Fig. 2, -●-○-, and -Δ-show the results obtained when the amount of SLO in a total reagents mixture (i.e., a mixture of a sample, a SLO solution and a liposome suspension) is 32 μg/ml, 16 μg/ml or 8 μg/ml, respectively. Fig. 3 (a) shows the result obtained in Example 4 and Fig. 3 (b), (c) and (d) those obtained in Example 5: (b), (c) and (d) shows the results obtained when the BSA content is 0.25%, 0.5% or 1.0%, respectively. In Fig. 3, the axis of abscissa refers to ASO value (Todd) and the axis of ordinate to absorbance at 410 nm. Fig. 4 shows the calibration curve obtained in Example 6: the axis of abscissa refers to ASO value (Todd) and the axis of ordinate to the change of absorbance per minute at 415 nm meesured by using as a reference a reaction solution using physiological saline in place of a sample. Fig. 5 shows the correlation between the present method (the method of Example 6) and a conventional method (the Rantz-Randall method).

DETAILED DESCRIPTION OF THE INVENTION

As the liposome used in this invention, any one may be used, and there may be exemplified, for example, those made of mixtures of phospholipids or glycolipids and cholesterols or other lipids, and those made of mixtures of these materials and lipopolysaccharides (hereinafter abbreviated as LPS) or LPS-like compounds. As the phospholipids, there may be used any of conventional ones, for example, alkyl ester type phospholipids such as dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), and the like; alkyl ether type phospholipids such as di-O-hexadecylphosphatidylcholine, di-O-octadecylphosphatidylcholine, didodecylphosphatidylcholine, and the like; and natural lecithins such as egg yolk lecithin, soybean lecithin, and the like. Among them, the alkyl ether type phospholipids are more preferable because in general they can form liposomes which are stable even at ordinary temperature.

As LPS, there are usually used those derived from E. coli, those obtained by treatment by the Westphal method, etc.

As the LPS-like compounds, there may be exemplified compounds obtained by subjecting LPS to chemical modification, for example, compounds obtained by oxidation of the saccharide chain portion, compounds obtained by chemical treatment such as acetylation, succinylation, phthalylation or the like of the saccharide chain portion, and complexes of LPS and polymyxin having a high affinity for LPS or a carbocyanine type dye which is a basic photosensitive dye, etc.

As the glycolipids, conventional ones may be exemplified, and it is also possible to use conventional lipids other than those described above.

As the liposome usd in this invention, liposomes containing LPS or a LPS-like compound as a part of their constituents are preferred because they make it possible to increase the amount of a labeling substance to be encapsulated. As for such liposomes, the specificaton of European Patent Application No. 87107259,1 which the present inventors have previously filed is referred herein.

The labeling substance to be encapsulated in the liposomes includes, for example, enzymes such as alkaline phosphatase, β-galactosidase, glucose-6-phosphate dehydrogenase, etc.; coenzymes such as NAD, NADP, FAD, etc.; luminous compounds such as luminol, luciferin, etc.; fluorescent substances such as carboxyfluorescein, etc.; dyes such as 2,7-bis(2-arsonophenylazo)-1,8-dihydroxynaphtalene-3,6-disulfonic acid, etc.; sugars such as glucose, etc.; ionic compounds such as potassium dichromate, sodium dichromate, NaCl, etc.; and radical compounds such as nitroxide compounds, etc. But the labeling substance is not limited thereto, and any substance may be used so long as it can be encapsulated in the liposomes and can serve as a marker in determination of ASO value.

In this invention, the enzymes are particularly preferred as the labeling substance.

As a method for preparing the liposomes used in this invention, conventional preparation methods may be exemplified. That is to say, there may be exemplified all the well known methods for preparing liposomes, for example, the voltexing method [A.D. Bangham et al., J. Mol. Biol., 13, 238 (1965)], sonication methods [C. Huing et al., Biochemistry, 8, 344 (1969)], surfactant method [J.R. Slack et al., Biochem. Biophys. Acta., 323, 547 (1973)], reverse-

phase evaporation method (REV method) [F. Szoka et al., Proc. Natl. Acad. Sci., U.S.A., 75, 4194 (1978)], ethanol infusion method, ether infusion method, pre-vesicle method [S.Batzri et al., Biochem. Biophs. Acta., 298, 1015 (1973)], French press extrusion method, Ca^{2+} fusion method, annealing method, freeze-thaw-fusion method, freeze-drying methods, W/O/W emulsion method, methods such as the stable plurilamellar vesicle method (SPLV method) recently reported by S.M. Gruner et al. [Biochemistry, 242833 (1985)], and methods for preparing liposomes called "giant liposomes" which have a large captured volume. The liposomes containing LPS or a LPS-like compound are prepared according to the process disclosed in the specification of European Patent Application No. 87107259,1.

The labeling substance to be encapsulated in the liposomes is usually added at an optional step in the above-mentioned method for preparing liposomes.

The determination method of this invention comprises adding a suspension of liposomes encapsulating the labeling substance and a solution of SLO as antigen to a sample; measuring the amount of the labeling substance released by lysis of the liposome membrane by SLO; and thereby determining ASO value in the sample.

The using amounts of the liposomes, labeling substance, SLO, etc. used in the determination method of this invention in terms of their concentration in a total reagents mixture, i.e., a mixture of a sample, a SLO solution and a liposome suspension (or a total reagents mixture before addition of the reaction terminator solution hereinafter described, in the case of determination using the reaction terminator) are as follows. The amount of the liposomes is usually 5 to 500 n mol/ml, preferably 30 to 100 n mol/ml in the total reagents mixture in terms of the amount of phospholipid or glycolipid. The amount of the labeling substance to be encapsulated in the liposomes is varied depending on the kind of the labeling substance, but for example, when the labeling substance is alkaline phosphatase (hereinafter abbreviated as AP), said amount is usually 0.02 to 0.8 unit/ml, preferably 0.1 to 0.2 unit/ml in the total reagents mixture. Although the amount of SLO is not critical because it is varied depending on the kind of the liposome, an amount of 0.5 to 100 μg/ml in the total reagents mixture is usually preferably employed. When an enzyme is used as the labeling substance in the determination method of this invention, a substrate is used for measuring the amount of the enzyme released from the liposomes. The amount of the substrate used is varied depending on the kinds of the enzyme and substrate used, but for example, when AP is used, said amount is usually 0.5 to 10 mM, preferably 2 to 5 mM in terms of the concentration of p-nitrophenylphosphate as substrate solution.

In the determination method of this invention, a buffer solution is usually used for conducting the determination stably or as a diluent for the liposome suspension, the SLO solution, etc. As the buffer solution, there can be used all the conventional buffer solutions including phosphate buffer, Good's buffer (HEPES, PIPES, MES, etc.), Tris buffer, etc. Water-soluble proteins such as bovine serum albumin (BSA), gelatin and the like, sugars, chelating agents, reducing agents, etc. may be properly added as additives to these buffer solutions. SLO is used usually in the form of an aqueous solution but is relatively unstable in an aqueous solution. Therefore, for stabilizing SLO, it is preferable to incorporate water-soluble proteins such as BSA, gelatin, etc. previously into the buffer solution used for the SLO solution. Although the adding amount of the protein is not critical, it is usually about 0.05 to about 2.0 w/v% in the buffer solution. In the determination method of this invention, ASO value can be accurately determined even when the above-mentioned proteins are added.

The determination method of this invention is described below, for example, by taking the case where liposomes encapsulating AP as a labeling substance are used and p-nitrophenylphosphate is used as a substrate.

First, a sample and SLO are mixed and then pre-incubated for about 1 to about 10 minutes, followed by adding thereto the liposomes; or a sample, SLO and the liposomes are mixed simultaneously. Then, the resulting mixture was incubated for 5 to 30 minutes, after which p-nitrophenylphasphate as substrate is added and the mixture thus obtained is incubated for another 5 to 30 minutes. After the predetermined time, the reaction is terminated by addition of a reaction terminator (e.g., NaOH, KOH, etc.), and absorbance at 410 nm is measured. Alternatively, all the reagents (i.e., SLO, the liposomes and the substrate) are allowed to act on a sample at the same time, and after incubation for 5 to 30 minutes, the reaction is terminated by addition of a reaction terminator and absorbance at 410 nm is measured. Subsequently, ASO value in the sample is determined from a calibration curve previously prepared by use of standard samples.

When a enzyme is used as a labeling substance, it is also possible to determine ASO value by measuring the change of absorbance per predetermined time after preincubating the total reagent mixture and a substrate or preincubating the total reagent mixture and thereafter adding the substrate.

Although either of the methods described

above may be employed, the latter method in which a sample, SLO and the liposomes are mixed simultaneously is superior because its procedure is simple and the determination can be carried out in a short time.

It is also possible to determine ASO value by measuring absorbance after substantial completion of the lysis of the liposomes without using a reaction terminator.

For example, when a luminous substance, a dye or the like is used as the labeling substance, the amount of the labeling substance released from the liposomes is determined by measuring absorbance directly without using a substrate. Also when other labeling substances are used, the amount of these substances released can be determined by a well known method for them.

As is clear from the above detailed description, the kit used for the determination method of this invention comprises a suspension of liposomes encapsulating a labeling substance and a solution of SLO.

The solution of SLO preferably contains water-soluble proteins such as BSA, gelatin, etc., particularly preferably BSA.

For example, when an enzyme or the like is used as the labeling substance, in addition to these reagents a substrate solution for the enzyme or the like is used.

The conventional buffer solutions described above also can optionally be added.

As described above in detail, the determination method of this invention is sufficiently applicable not only to a manual method but also to determination using an automatic analyzer, and permits easy and rapid determination. When the determination is carried out by means of an automatic analyzer, combination of reagents, etc. are not critical and there may be properly selected a combination of reagents which seems to be the most suitable in consideration of the type of the automatic analyzer or other factors.

This invention is further explained in more detail with reference to Referential Examples and Examples, which are not by way of limitation but by way of illustration.

Referential Example 1

Preparation of liposomes encapsulating AP using di-O-hexadecylphosphatidylcholine as a phospholipid

In a test tube were placed 325 µl of a 20 mM solution of di-O-hexadecylphosphatidylcholine in chloroform, 325 µl of a 20 mM solution of cholesterol in chloroform, and 85 µl of a 6 mM solution of dipalmitoylphosphatidylglycerol in chloroform/methanol (95:5), and after mixing, the solvent was distilled off by means of a rotary evaporator. The residue was dried in a desiccator for 2 hours, after which 0.5 ml each of chloroform and diethyl ether were added, followed by adding thereto 80 µl of an alkaline phosphatase (AP) solution [a solution of 5,000 unit/2.5 ml of Ap mfd. by Sigma Chemical Company in 0.01M HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid) buffer, pH 7.4], and the resulting mixture was vigorously stirred with a Vortex mixer. The mixture was then concentrated on a water bath at 43° to 48°C by means of a rotary evaporator, and the organic solvent was removed by distillation, after which 1 ml of 0.01M HEPES buffer was added and the resulting mixture was stirred with a Vortex mixer until uniform dispersion was achieved. The dispersion thus obtained was transferred to a centrifuge tube and centrifugation at 34,000 r.p.m. at 4°C for 40 minutes was repeated 5 times to remove the supernatant. Then, the pellet (the precipitation) was suspended in 2 ml of 0.01M HEPES buffer and the resulting suspension was stored at 4°C.

Referential Example 2

Preparation of liposomes encapsulating AP using LPS as a part of constituents

LPS-liposomes encapsulating AP were prepared in exactly the same manner as in Referential Example 1, except that a mixture of the lipid solutions used in Referential Example 1 and a suspension of 0.5 mg of lipopolysaccharide (LPS) in 1 ml of chloroform/methanol (1:1) was used in place of said lipid solutions.

Referential Example 3

Preparation of liposomes encapsulating AP using LPS and DPPC

In a round bottom flask were placed 2 ml of 20 mM solution of dipalmitoylphosphatidylcholine (DPPC) in chloroform and 2 ml of a 20 mM solution of cholesterol in chloroform, and after mixing, the solvent was distilled off by means of a rotary evaporator. Further, the residue was dried in vacuum by means of a vacuum pump in a desiccator for 2 hours to form a thin film of the lipids on the interior surface of the flask. The thin film was hydrated by addition of 0.6 ml of a 200 mM aqueous n-octyl glucoside solution containing LPS in an amount of 0.6 mg/ml and 0.01M HEPES buffer (pH 7.4), and the mixture thus obtained was stirred with

a Vortex mixer until uniform dispersion was achieved. Then, 250 1 of an aqueous AP solution (2,000 unit/ml) was added, and after stirring and mixing for a while, the resulting mixture was dialyzed against 0.01M HEPES buffer at 50°C. After about 2 hours, the liposome suspension thus obtained was transferred to a centrifuge tube, and centrifugation at 35,000 r.p.m. at 4°C for 40 minutes was repeated 5 times to remove the supernatant. Then, the pellet was suspended in 0.01M HEPES buffer and the resulting suspension was stored at 4°C.

Reperential Example 4

Preparation of liposomes encapsulating AP using LPS and egg yolk lecithin

Liposomes encapsulating AP were prepared in exactly the same manner as in Referential Example 3, except that a 1.4 wt% solution of egg yolk lecithin in chloroform was used in place of the DPPC solution and that the temperature at dialysis was changed into from 50°C to room temperature.

Referential Example 5

Preparation of liposomes encapsulating AP using LPS, DPPC and egg yolk lecithin

Liposomes encapsulating AP were prepared in exactly the same manner as in Referential Example 3, except that 1.5 ml of a 1.4 wt% solution of egg yolk lecithin in chloroform and 0.5 ml of a 20 mM solution of DPPC in chlorotorm were used in place of the DPPC solution and that the amount of the aqueous AP solution was changed into from 250 µl to 1.5 ml.

Example 1

To 10 ul of the liposome suspension obtained in Referential Example 1 were added 20 µl of each of ASO solutions different in concentration, 20 µl of a SLO solution (concentration: 1 mg/ml) and 400 µl of a 2 mM p-nitrophenylphosphate disodium solution and the resulting mixture was incubated at 37°C for 30 minutes. Then, 1.5 ml of a 0.1N NaOH solution was added to terminate the reaction and absorbance at 410 nm was measured. The result obtained is shown by -O-in Fig. 1.

The amounts of the liposomes, AP and SLO in the total reagents mixture (which did not contain the NaOH solution) were 60 n mol/ml in terms of the amount of phospholipids, 0.6 unit/ml and 44.4 µg/ml, respectively. As is obvious from Fig. 1, a good linear relationship exist between ASO value and absorbance, indicating that ASO value can be accurately determined by this invention.

Example 2

Determination was carried out in exactly the same manner as in example 2, except that 10 µl of the LPS-liposome suspension obtained in Referential Example 2 was used, to obtain the result shown by -●-in Fig. 1.

Example 3

To 20 µl of each of ASO solutions different in concentration was added 20 µl of the liposome suspension obtained in Referential Example 3, and the resulting mixture was diluted to a volume of 200 µl with 50 mM Tris [tris(hydroxymethyl)-aminomethane]-HCl buffer (pH 7.8). 20 µl of a SLO solution (predetermined concentration) and 400 µl of 2 mM p-nitro-phenylphosphate solution were added to the resulting dilute. After incubation at 37°C for 20 minutes, the reaction was terminated by addition of 2 ml of a 0,1N NaOH solution and absorbance at 410 nm was measured. The relationship between ASO value (Todd) and relative absorbence (a value determined by taking the absorbance measured for a sample containing no ASO as 100) is shown in Fig. 2. In Fig. 2, -●-. -O-and -Δ-show the results obtained when an SLO solution having a concentration of 1 mg/ml, 0.5 mg/ml or 0.25 mg/ml, respectively, was used, that is to say, -●-. -O-and -Δ-show the result obtained when the amount of SLO in the total reagents mixture was 32 µg/ml, 16 µg/ml or 8 µg/ml, respectively.

Exampel 4

10 µl of the liposome suspension obtained in Referential Example 4 was diluted in 20 times with 50 mM Tris-HCl buffer containing 0.1% BSA and gelatin (pH 7.8). To 200 µl of the resulting dilution was added 400 µl of 2 mM p-nitrophenylphosphate disodium solution containing SLO (the amount of SLO: 2 µg) obtained by dilution with the same buffer as described above, and the resulting mixture was incubated at 37°C for 30 minutes. Then 2 ml of a 0.1N NaOH solution was added to terminate the reaction and absorbance at 410 nm was measured. The result obtained is shown in Fig. 3-(a).

Example 5

Determination was carried out in exactly the same manner as in Example 4, except for changing the BSA content of the buffer solution used for diluting the liposome suspension and the substrate solution containing SLO. The results obtained are shown in Fig. 3 (b), (c) and (d). In Fig. 3, (b), (c) and (d) shown the results obtained when the BSA content was 0.25%, 0.5% or 1.0%, respectively.

It can be seen that as is clear from these results, the determination method of this invention is not adversely affected by the content of BSA which was added for stabilizing SLO in the diluting buffer solution.

Example 6

ASO value was determined by means of an automatic analyzer Hitachi Model 7050.

First, 5 µl of a sample was mixed with 350 µl of a first reagent R1 [prepared by diluting 2 µl of the liposome suspension obtained in Referential Example 5 to a volume of 350 µl with 50 mM Tris-HCl buffer (containing 0.17% BSA and gelatin)] and correction blank was conducted. After 5 minutes, 250 µl of a second reagent R2 (the amount of SLO: 0.5 µg, substrate concentration: 2 mM) was added. After another 5 minutes, change of absorbance per minute at 415 nm (700 nm) was measured. A calibration curve for ASO value in the present method is shown in Fig. 4. In Fig. 4, the axis of abscissa refers to ASO value (Todd) and the axis of ordinate to the change of absorbance per minute at 415 nm measured by using as a reference a physiological saline in place of a sample. The correlation between the present method and a conventional method (Rantz-Randall method) is shown in Fig. 5.

As is obvious from Fig. 5, the values obtained by the determination method of this invention correlates well to those obtained by the conventional method, and therefore this invention permits accurate determination.

Claims

1. A method for determination of antistreptolysino (ASO) value, which comprises

adding liposomes encapsulating a labeling substance and streptolysino (SLO) as both antigen and lytic agent to a sample,

measuring the amount of the labeling substance released by lysis of the liposome membrane by SLO, and

thereby determining ASO value in the sample.

2. The method according to Claim 1, wherein the labeling substance is selected from the group consisting of enzyme, coenzymes, luminous compounds, fluorescent compounds, dyes, sugars, ionic compounds and radical compounds.

3. The method according to Claim 1, wherein the labeling substance is an enzyme.

4. The method according to Claim 2, wherein the determination is carried out in the presence of a water-soluble protein.

5. The method according to Claim 4, wherein the water-soluble protein is bovine serum albumin (BSA).

6. The method according to Claim 1, wherein the liposome contains a lipopolysaccharide (LPS) or a LPS-like compound as a part of its constituents.

7. A method for determination of ASO value, which comprises

adding a suspension of liposomes encapsulating a labeling substance and a solution of SLO to a sample,

measuring the amount of the labeling substance released by lysis of the liposome membrane by SLO remaining after the antigen-antibody reaction between ASO in the sample and SLO added, and

determining ASO value in the sample from a standard calibration curve previously prepared by conducting determination for standard samples in the same manner as described above.

8. The method according to Claim 7, wherein the amount of the lipsomes used for the determination is 5 to 500 n mol/ml in terms of phospholipid or glycolipid in a total reagents mixture (a mixture of a sample, a solution of SLO and a suspension of liposome), and the amount of SLO used therefor is 0.5 to 100 µg/ml in the total reagents mixture.

9. The methods according to Claim 7, wherein the solution of SLO contains a water-soluble protein.

10. A kit for determining ASO value in a sample which comprises a suspension of liposomes encapsulating a labeling substance and a solution of SLO.

11. The kit according to Claim 10, wherein the labeling substance is an enzyme.

12. The kit according to Claim 10, which further comprises a substrate solution.

13. The kit according to Claim 10, wherein the solution of SLO contains a water-soluble protein.

14. The kit according to Claim 10, wherein the liposome contains a lipopolysaccharide (LPS) or a LPS-like compound as a part of its constituents.

FIG. 1

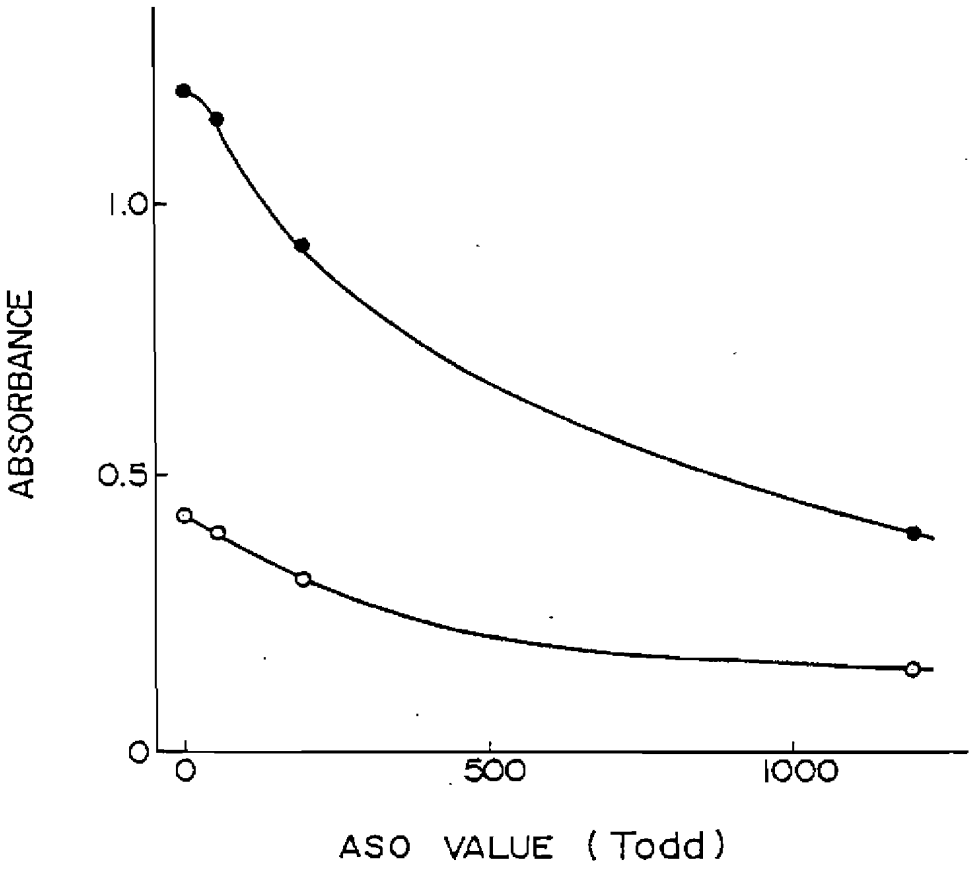


FIG. 2

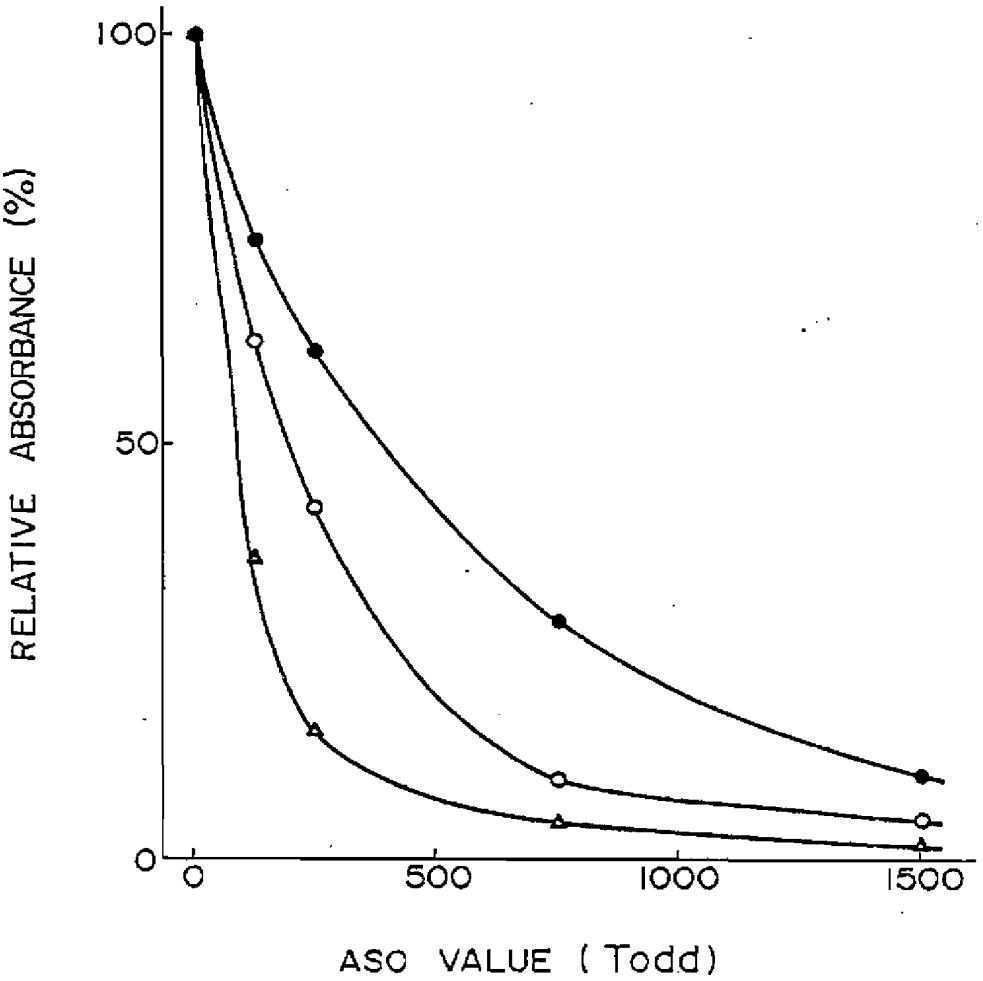


FIG. 3

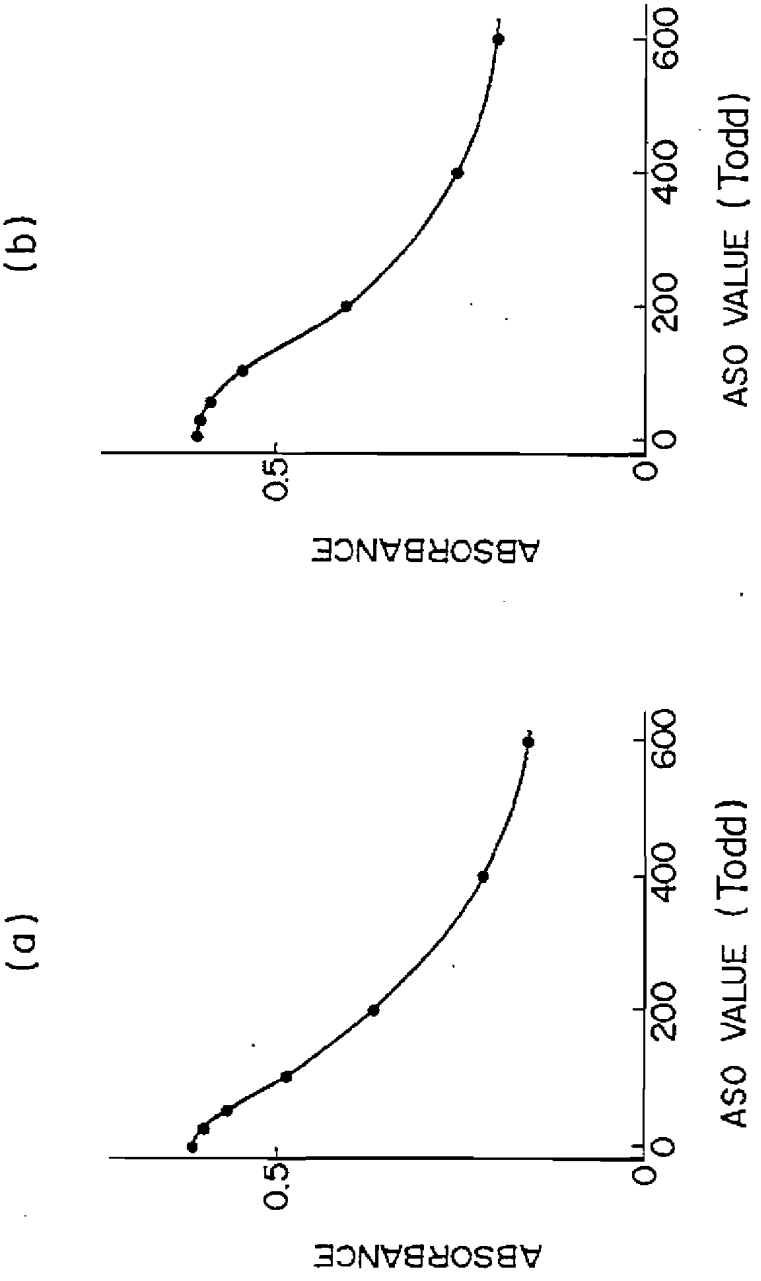

(a)
ABSORBANCE
0.5
0
0
200
400
600
ASO VALUE (Todd)
(b)
ABSORBANCE
0.5
0
0
200
400
600
ASO VALUE (Todd)

FIG. 4

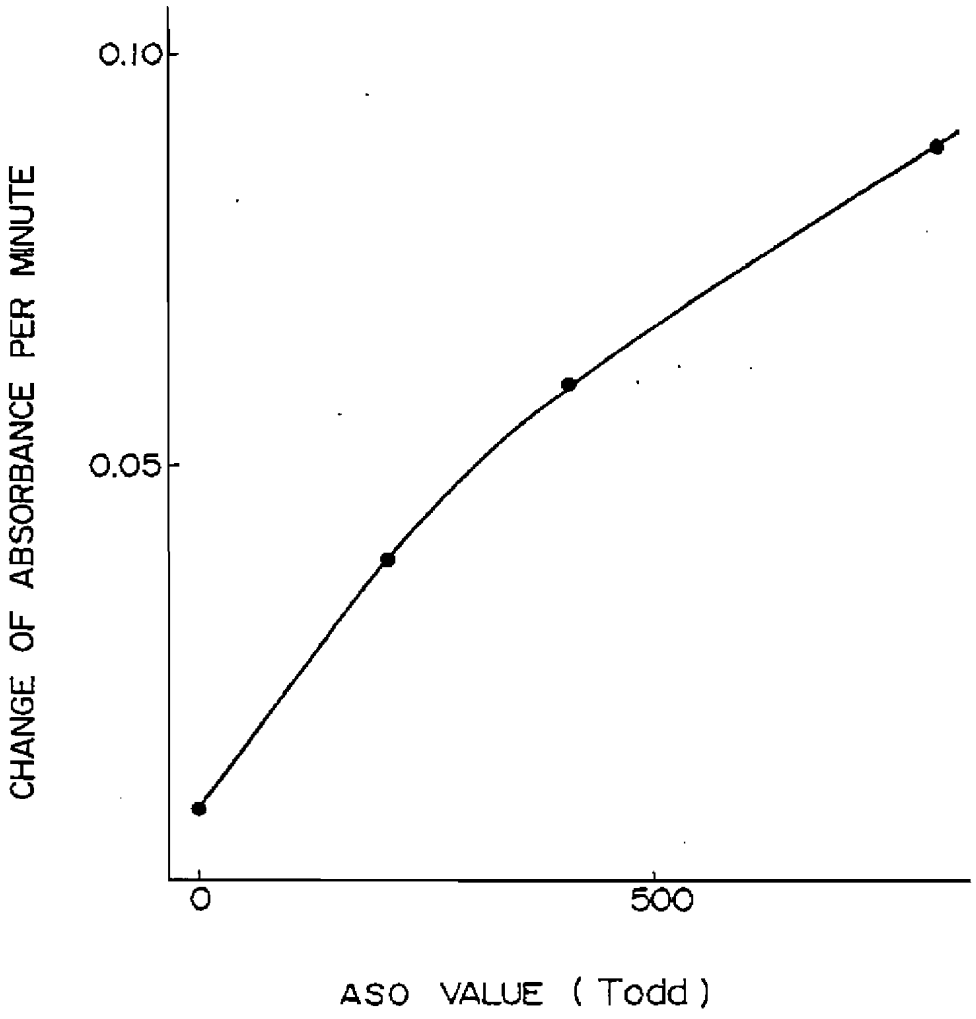
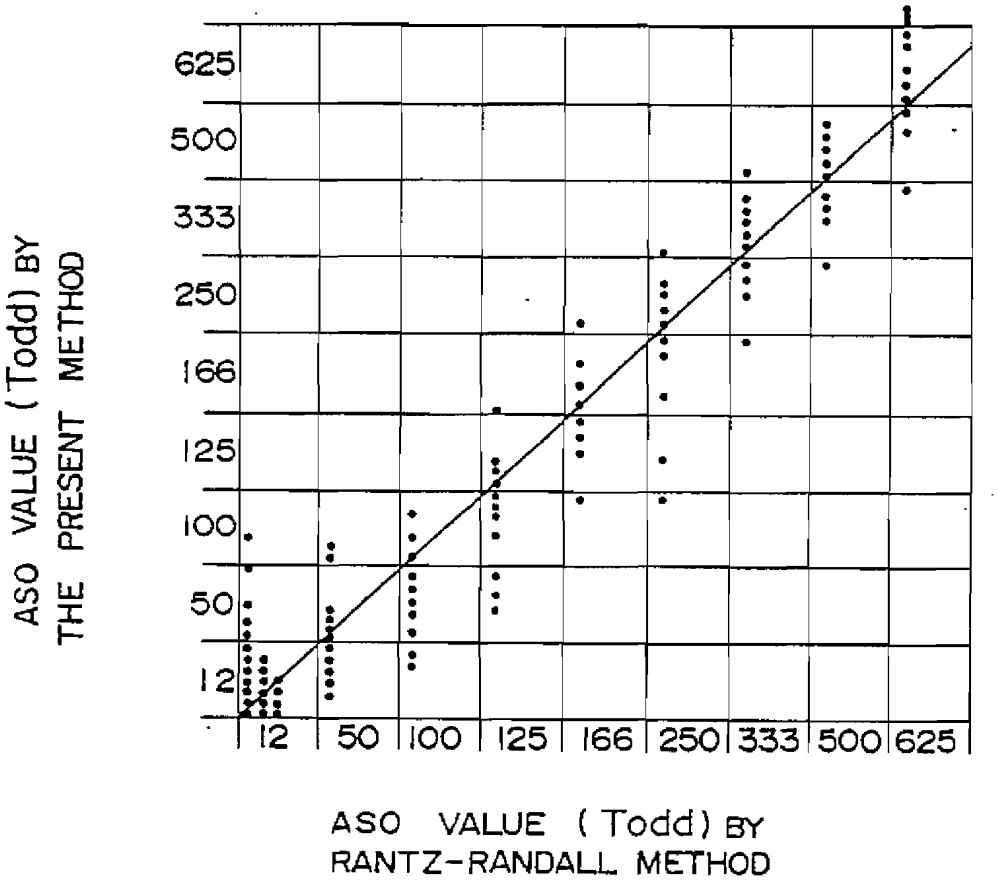


FIG. 5


ASO VALUE (Todd) BY THE PRESENT METHOD
625
500
333
250
166
125
100
50
12
12
50
100
125
166
250
333
500
625
ASO VALUE (Todd) BY RANTZ-RANDALL METHOD

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 87 11 3783

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.3)
Y	GB-A-2 069 133 (COLLABORATIVE RESEARCH) * complete * ---	1,2,7, 11	G 01 N 33/569// G 01 N 33/546 G 01 N 33/549
Y	EP-A-0 043 608 (DIESSE DIAGNOSTICA SENESE) * page 2, line 26 - page 3, line 32; page 10, claim 1 * ---	1,2,7, 11	
A	DE-B-2 657 926 (ISTITUTO SIEROTERAPICO E VACCINOGENO TOSCANO SCLAVO S.P.A.) * c olumn 1, claim * ---	1	
A	EP-A-0 108 612 (AMERICAN HOME PRODUCTS) * page 3, line 1 - page 4, line 27; page 13, claims 1, 2 * ---	1	
A	EP-A-0 089 598 (BEHRINGWERKE AG) * page 1, line 28 - page 2, line 15; page 8, claims 1, 2 * ---	1	
A	CHEMICAL ABSTRACTS, vol. 90, no. 2, February 1979, page 87, column 2, abstract no. 6712ls, Columbus, Ohio, US; D. PRIGENT et al. "Interaction of streptolysin O with liposomes", & LABO-PHARMA - PROBL. TECH. 1978, 26(281), 910-913 --- -/-	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.3)

G 01 N 33/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	21-01-1988	GREEN C.H.

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L : document cited for other reasons

& : member of the same patent family, corresponding document

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DOCUMENTS CONSIDERED TO BE RELEVANT

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A	CHEMICAL ABSTRACTS, vol. 83, no. 21, 24th November 1975, page 131, columns 1, 2, abstract no. 173595q, Columbus, Ohio, US; J.L. DUNCAN et al.: "Effect of streptolysin O on erythrocyte membranes, liposomes and lipid dispersions. Protein-cholesterol interaction", & J. CELL. BIOL. 1975, 67(1), 160-173	1	

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